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VEGF Induced Differentiation of Gingival Stem Cells to Endothelial Cells in Vitro

Garima Gupta
Nova Southeastern University

VEGF INDUCED DIFFERENTIATION OF GINGIVAL STEM CELLS TO ENDOTHELIAL CELLS *IN VITRO*

BY

GARIMA GUPTA, D.D.S.

A Thesis Presented to the Faculty of the College of Dental Medicine of

Nova Southeastern University in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE IN DENTISTRY

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I certify that I am the sole author of this thesis, and that any assistance I received in its preparation has been fully acknowledged and disclosed in the thesis. I have cited any sources from which I used ideas, data, or words, and labeled as quotations any directly quoted phrases or passages, as well as providing proper documentation and citations. This thesis was prepared by me, specifically for the M.Sc.D. degree and for this assignment.

STUDENT SIGNATURE: _____

Garima Gupta, D.D.S. Date

DEDICATION

To my loving husband Rajesh, adorable son Rayan, my wonderful parents and the best parents-in-law for all of your support and understanding me throughout my journey. Thank you for always standing by me to achieve my goals.

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ABSTRACT

VEGF INDUCED DIFFERENTIATION OF GINGIVAL STEM CELLS TO ENDOTHELIAL CELLS *IN VITRO*

DEGREE DATE: JUNE 21, 2019 Garima Gupta, D.D.S.

COLLEGE OF DENTAL MEDICINE NOVA SOUTHEASTERN UNIVERSITY
Directed by: Dr. Umadevi Kandalam, Associate Professor, Department of Pediatric Dentistry, NSU College of Dental Medicine.

Background: Birth defects that result in structural malformation of major organs are one of the major causes of mortality in children. Tissue engineering and regenerative medicine (TERM) strategies are particularly beneficial for the pediatric patients to correct these defects owing to their greater regenerative capacity compared to the adult population. Limited availability of pediatric patient's auto-transplantation of organs makes TERM strategies as a promising alternative to conventional clinical procedures that utilize the grafts. Recent developments in stem cells technologies, there is an enormous potential for TERM in correcting birth defects and enhancing the life span in young population. However, the major challenge to the craniofacial bone tissue engineering is the repair and regeneration of critical size bone defects accompanied by functional vascular network. Insufficient vasculature in regenerated bone is the main causes of large graft failure, leading to inner graft necrosis and lack of integration with the host tissue. While, it is well established that human gingiva derived mesenchymal stem cells (GMSCS) are robust source of osteogenic precursors in bone regenerative TERM, it is unknown if GMSCS also can differentiate into endothelial cells. The aim of this study was to explore the potential of GMSCS to differentiate into endothelial cells.

Methods: GMSCs were cultured under standard conditions. The angiogenic differentiation was induced administering Vascular Endothelial Growth Factor (VEGF). The expression of endothelial marker gene expression was measured by using quantitative PCR method. A functional tube formation assay was performed to assess the endothelial differentiation. Immunofluorescence was conducted to detect the surface marker protein CD31.

Results: Our results showed a dose dependent increase in the expression of the endothelial marker genes, PECAM-1, VCAM-1, KDR, FLT-1 and PCDH12. The tube formation assay revealed the ability to form capillary structures in the cells induced with 10 and 50ng/ml VEGF. The data on Immunofluorescence demonstrated the distinct presence of CD31 surface marker.

Conclusions: Our results showed that GMSCS have the potential to differentiate into endothelial cells. Thus, they serve as sources for programmed angiogenic and osteogenic cells to contribute for the regeneration of the vascularized bone to make a paradigm-shift in stem cell therapy.

Key words: Gingiva derived stem cells, Vascular endothelial growth factor, Endothelial cells, Angiogenic differentiation, Craniofacial defect

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ABBREVIATIONS

HGMSCs	Human gingival derived mesenchymal stem cells
DMEM	Dulbecco's modified eagle medium
VEGF	Vascular Endothelial Growth factor
EPC	Endothelial Progenitor cells
HUVECs	Human Umbilical Vein Endothelial Cells
PCDH12	Protocadherin 12
PECAM1	Platelet endothelial cell adhesion molecule-1
VECAM1	Vascular cell adhesion molecule-1
VEGFR	Vascular Endothelial Growth Factor Receptor
TERM	Tissue engineering and regenerative medicine

CHAPTER 1 INTRODUCTION

1.1 Craniofacial Bone Formation Key Considerations

Bone is a dynamic and highly vascularized tissue that contains extracellular matrix composed of inorganic and organic elements that contains a number of types of cell that are responsible for its metabolism.[1] The formation of bone is multi-step process with the interactions among various cells, extracellular matrix and organic and inorganic molecules. In particular, during craniofacial bone development, many biophysical forces, particularly mechanical load and locally released growth factors are important regulatory factors. Additionally, craniofacial skeleton involved in specific function such as protection of brain and optic tract, while facilitating mastication.[2]

1.2 Clinical Relevance

Birth defects that result in structural malformation of major organs are one of the major causes of mortality in children. Craniofacial bone defects due to congenital anomalies, cancers, and trauma and fractures are most common. While the birth defects with congenital malfunctions are 2-3%, the prevalence of craniofacial fractures in pediatric population is 58% of total fractures annually. While treatment of cranial vaults needs permanent protection, reconstruction of segmental defects requires restoration of mechanical integrity and temporal joint function. Unlike adult patients, reconstruction of these mandibular and maxillo-facial bones in growing babies require a proper understanding of the changes in the bone architecture to

achieve optimal restoration of mastication, deglutition, and cosmetic issue. Thus, the medical cost associated to repair of these defects, in particular the critical size defects are over $2.5 billion/annum.[3,4]

1.3 Critical Size Defects and Current Treatment Modalities

A critical size bone defect is a large void in a bone beyond a critical healing threshold, which cannot heal spontaneously. Attempting the repair in the absence of bone grafts can impede the healing processes accompanied by osteogenesis, which results in formation of fibrous connective tissue rather than bone warrants the need of bone grafts.[5] Currently, 2.2 million bone graft procedures are performed around the world each year to repair the critical size defects.[3] The current gold standard treatment for reconstruction of critical size bone defect in the craniofacial region is the implantation of autologous bone graft. Bone graft from extra oral surgical harvest site commonly includes tibia and ilium. However, for pediatric patients, graft harvest from autologous sites increases the additional risk and morbidity of a second surgical procedure, the donor site morbidity and low availability. Allografts, on the other hand, are concerned of risk for viral infections. In addition, there is increased unpredictable outcome using the bone grafts, when bone defect size is greater than 4 cm.[6] All these drawbacks associated with grafts call for the development of stem cell-based tissue engineering strategies as a promising alternative way to regenerate bone.[4,7-11]

1.4 Bone Tissue Engineering

Human stem cell-based tissue engineering emerged as a viable option for the regeneration of bone even in large craniofacial skeletal defects. There are three

mains components of the “golden triad” of tissue engineering which includes cells, growth factors and scaffolds[6] (Figure 1). However, the challenges in bone tissue engineering include induction of proper vasculature at the defect site in the regenerated bone tissue.

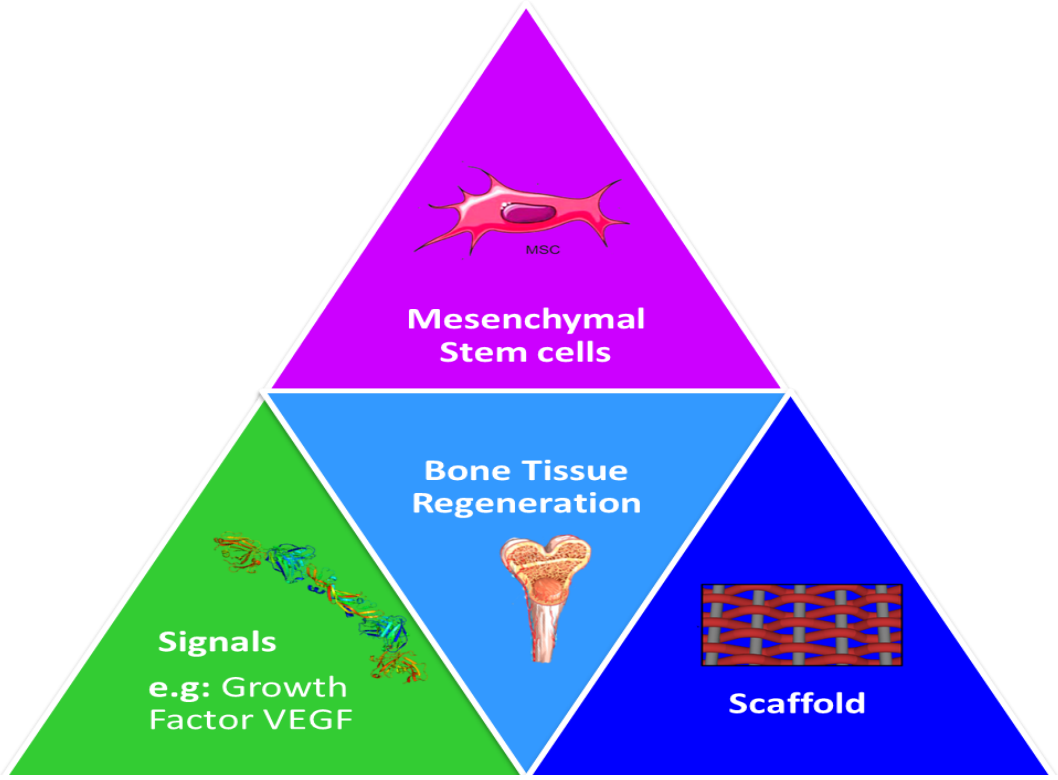


Figure 1: Essential Components of Tissue Engineering

1.5 Significance of Vascularization in Bone Regeneration

The clinical success of bone tissue engineering lies on developing a functional/vital bone with efficient vascularization. Bone formation indeed can progress successfully if there is adequate vascularization at the site. In addition to the bone formation, vasculature is also essential for bone remodeling during fracture healing and therefore, angiogenesis is a key component in bone repair.[12,13] Defective angiogenesis and any alteration in the vascular supply can result in osteonecrosis,

osteomyelitis and delayed fracture healing or non- union.[12,14] Osteonecrosis or avascular necrosis happens when there is inadequacy of blood supply to the bone, leading to death of bone cells. Osteomyelitis is an infection within the bone, which is mainly caused by poor vascularization.[12] Thus, bone vasculature is not only essential for regeneration, remodeling and homeostasis but is also essential for maintaining the functional integrity and longevity in the host.[13-16] While the gold standard autologous bone grafts have a pre-existing vascular network that meets the demand of oxygen and nutrients supply, the engineered grafts are, in general, incapable of generating sufficient vasculature. Vascularization supports bulk transport of nutrients and convective oxygen transport. However, although spontaneous vascularization does happen in the host to the graft, the vessel in-growth is too slow to provide adequate nutrient and oxygen transport to the cells in the deeper regions. Therefore, the engineered grafts have to rely mainly on passive diffusion of nutrient and oxygen, which is often insufficient, resulting in graft failure. Several strategies have been experimented to enhance vascularization including pre-vascularization of scaffolds and utilizing two co-culture methods, where endothelial cells will be co-cultured with stem cells.

1.6 Currently Available Techniques to Induce the Vascularization in Regenerating Bone Tissue

There are numerous methods to increase vascularization in bone tissue engineering; 1) combination of scaffold and angiogenic growth factors, and 2) *ex-vivo* pre-vascularization (i.e. co-culture of endothelial and osteogenic cells) technique. However, both techniques have pros and cons, as shown below

1.6.1 Combination of Scaffold and Angiogenic Growth Factors

A method of increasing vascularization in bone regeneration engineering by scaffold combined with angiogenic growth factors (VEGF, FGF). Hydrogels and other soft materials have been developed as suitable matrix materials for osteogenesis and angiogenesis due to their characteristic of loading with cells at relatively high density and retaining cell viability for longer period.[17] Nonetheless, there are some drawbacks associated with this technique, including, disruption of hydrogel structure and poor integration with the host tissue.[6] Although, incorporation of the angiogenic growth factors onto the scaffold has been shown to accelerate vascularization, it has been considered as a relatively inefficient process for bone formation, and presents the difficulty in selecting proper dosage of growth factor onto the scaffold with co-culturing between mature endothelial cells and osteogenic cells. These shortcomings include limited *in vitro* expansion of the endothelial cells.[2] It is also concerned if multiple different cell types can grow in concert to develop a structured bone tissue in the same *ex-vivo* culture. An another approach in the co-culture is to use different ratios of each cell type, but the main problem in this technique is to explore a way to identify different cell types.[7]

1.6.2 An *ex-vivo* Pre-vascularization (i.e., Co-Culture of Endothelial and Osteogenic Cells)

This technique utilizes co-culture of mature endothelial cells and osteogenic cells. Although mature differentiated endothelial cells may be used for bone tissue regeneration in combination of osteogenic cells, there are few pitfalls in co-

culturing between mature endothelial cells and osteogenic cells. These shortcomings include limited capacity to expand endothelial cell *in vitro*.[2] It is also concerned if multiple different cell types can grow in concert to develop a structured bone tissue in the *ex-vivo* co-culture. Another approach in the co-culture is to use different ratios of each cell type, but the main problem in this technique is to explore a way to identify different cell types.[7]

1.7 Proposed Method to Induce Vascularization (GMSCs to Endothelial cells)

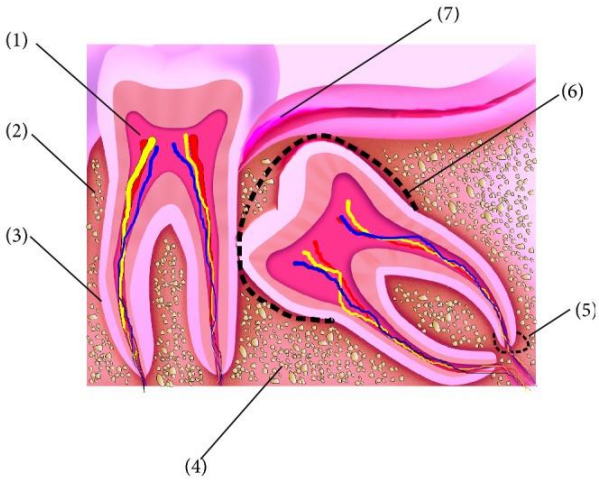
Based on the possible drawbacks of aforementioned techniques, we proposed in this study to differentiate mesenchymal stem cells derived from human gingiva (GMSCs) into endothelial progenitor cells (EPC) via stimulation with vascular endothelial growth factor (VEGF). In that way, it will reduce the drawbacks associated with transplantation of ex vivo co-cultured osteoblasts and endothelial cells in a scaffold, which require appropriate ratio of both cell types and other techniques using specially designed scaffolds.[2] The endothelial progenitor cells (EPC) are the ones with enhanced proliferative capacity and generates all subsets of endothelial cell lineage upon differentiation. It has been found that EPC have a higher survival rate as compared to mature endothelial cells (most commonly used human umbilical vein endothelial cells, HUVECs).[7] Furthermore, EPC have been shown to be 10 times more proliferative than HUVECs.[12]

The stem cell source that contains both osteogenic and endothelial progenitor cells is considered to be the most advantageous cell source.[2] Our lab has already established the osteogenic potential of GMSCs. By this method, it would be possible to use gingival stem cells as the cellular source for both osteogenic and

vasculogenic progenitor cells, rather than adding exogenous endothelial and osteoblast cell types to enhance angiogenesis during bone regeneration.

1.8 Gingival Mesenchymal Stem Cells (Cells Used in This Study)

In this study, we chose to use GMSCs. These cells are of special interest as they serve a promising cellular source especially in craniofacial tissue regeneration. 90% of the GMSCs are derived from cranial neural crest cells and they differentiate into cartilage and bone to form vast majority of craniofacial structures.[4,13,14] Moreover, mesenchymal stem cells deriving from gingival tissues are minimally invasive and are easily accessible[18-22] (Figure 2). GMSCs have the ability to proliferate faster than bone marrow stem cells, exhibits a stable morphology even after extended passaging and have potent immunomodulatory characteristics.[18,19] Furthermore, neural crest originated stem cells like GMSCs might be more efficient in regenerating bone in craniofacial region, when compared to the bone marrow derived stem cells and may show better tissue acceptability than MSCs from other tissues.[13,14,22-25]



(1) DPSCs and SHEDs
(2) Alveolar bone proper-derived stem cells
(3) PDLSCs
(4) BM-MSCs
(5) SCAP
(6) DFSCs
(7) G-MSCs

Figure 2: Sources of oral stem/progenitor cells isolated. DFSCs: dental follicle stem cells, G-MSCs: gingival mesenchymal stem/progenitor cells, PDLSCs: periodontal ligament stem cells, SHEDs: stem cells from the human exfoliated deciduous teeth, DPSCs: dental pulp stem cells, BM-MSCs: bone marrow mesenchymal stem cells, and SCAP: stem cells from the apical papilla (Reference: Fawzy El-Sayed KM, Dörfer CE. Gingival Mesenchymal Stem/Progenitor Cells: A Unique Tissue Engineering Gem)

1.9 Growth Factors

Growth factors are biological macromolecules which play an important role in regulating cell growth, differentiation and migration by targeting specific cellular receptors. Angiogenic growth factors are commonly expressed in response to injury and are produced by inflammatory cells and stromal cells to stimulate growth of blood vessels. There are several angiogenic growth factors that include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), tumor necrosis

growth factor (TNF-α), transforming growth factor beta (TGF-β), and the angiopoietins. Among these, the most commonly considered proangiogenic factor is vascular endothelial growth factor (VEGF).[6,13,26,27]

1.10 VEGF

The VEGF family consists of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, PIGF. All of the 7 members share common homology domain. VEGF original name was vascular permeability factor as it increases microvascular permeability and fenestration. VEGF has multiple functions in conditioning of angiogenesis as well as during angiogenesis processes. It stimulates proliferation, migration of endothelial cells which results in the formation of new blood vessels. Furthermore, studies have a shown that VEGF also facilitates tube formation and engages in differentiation of endothelial progenitor cells.[6,13,26,27]

1.11 Endothelial Cells

Studies have shown that endothelial cells can enhance bone regeneration ability. Endothelial cells are the key cellular element that compose blood vessels and capillaries (Figure 3). Endothelial cells are directly related to angiogenesis and provide the pre-requisite for bone regeneration.[7]

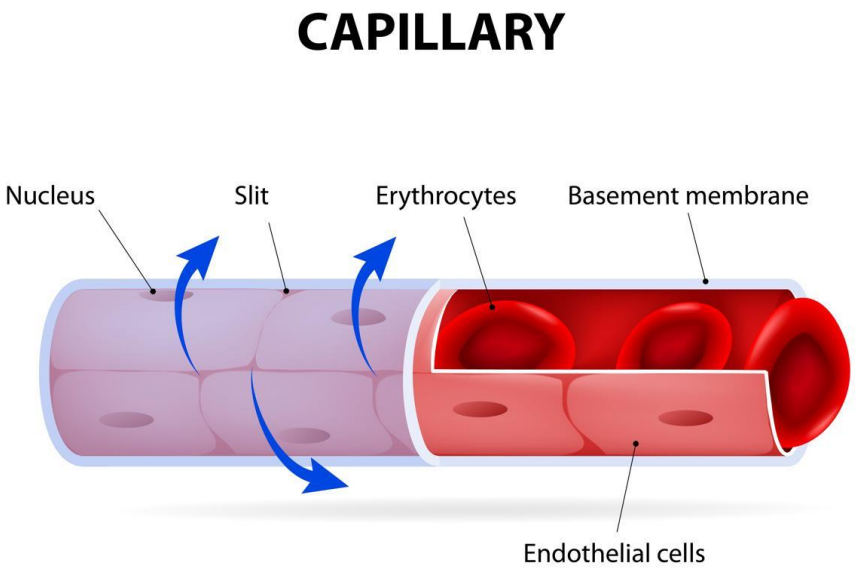


Figure 3: Endothelial cells

1.12 Mechanism how VEGF Stimulates Endothelial Cell Formation

VEGF is an angiogenic growth factor that works by binding with its two- cognate receptor type tyrosine kinases, VEGFR1, VEGFR2 which, in turn, elicits cell signals to induce angiogenesis.[13]

VEGF activates the phosphorylation of histone deacetylase 7 (HDAC7) via a PKC/PKD1 pathway. HDAC7 is mainly restricted in the nucleus of endothelial cells. HDAC7 phosphorylation stimulates nuclear export of HDAC7, which leads to activation of VEGF responsive genes. RCAN2 and Nur77 are the VEGF regulated genes required for migration and proliferation of endothelial cells.[28] In summary, VEGF signaling activates phosphorylation of HDAC7 and it regulates the expression of VEGF responsive genes involved in angiogenesis. [28] (Figure 4)

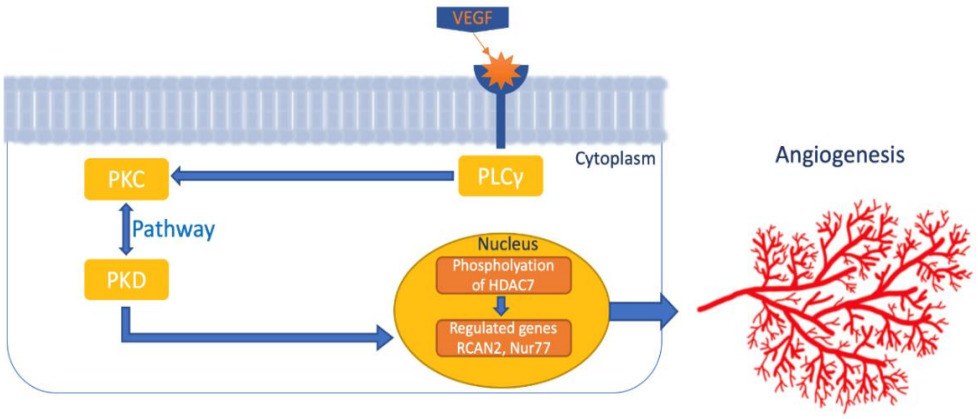


Figure 4: Mechanism of VEGF stimulating angiogenesis (Reference: Wang S, Li X, Parra M, Verdin E, Bassel-Duby R, Olson EN. Efficient Differentiation of Bone Marrow Mesenchymal Stem Cells into Endothelial Cells in Vitro)

1.13 Endothelial Marker Genes Used in This Study

The angiogenic differentiation of gingival stem cells were identified by the expression of these endothelial marker genes: VCAM-1, PCDH12, VEGF receptors (FLT1 and KDR), PECAM-1. Following are the endothelial markers that have been evaluated.

1.13.1 Vascular Cell Adhesion Molecule-1(VCAM-1)/ CD106

This is the main endothelial receptor that plays an important role in mediating adhesion of leukocytes to vascular endothelium. In addition to that, it also engages in regulation of T-lymphoblast movement through endothelial venular walls. Hence, it elicits cell signal transduction in between leukocyte and endothelial.[29]

1.13.2 Protocadherin 12 (PCDH12)/ VE cadherin

PCDH12 is a transmembrane protein mainly located at intercellular junctions. Intercellular junctions have been shown to control some part of the endothelial permeability to plasma proteins and circulating cells. Its expression is characteristically noticed in endothelial cells. It is mainly responsible for calcium dependent cell to cell adhesion.[30,31]

1.13.3 VEGF Receptors

1.13.3a FLT1/ VEGFR-1

VEGF regulates endothelial cell cycle through modulating molecular signal pathways. The most significant effect of VEGF receptor-1 is controlling the cell migration by regulating actin reorganization via the activation of p38 MAK kinase. In addition to that, it also triggers activation and movement of monolayer phagocytes across an endothelial cell monolayer. This interaction generates chemotactic response in polymorphonuclear cells.[26,32,33] (Figure 5)

1.13.3b KDR/ VEGFR-2

KDR promotes cell migration by controlling cell adhesion and mediates DNA synthesis of endothelial cells via the activation of ERK1/2. Furthermore, it also enhances vascular permeability during angiogenesis by stimulating the synthesis of Platelet activating factor (PAF).[26,32,33] (Figure 5)

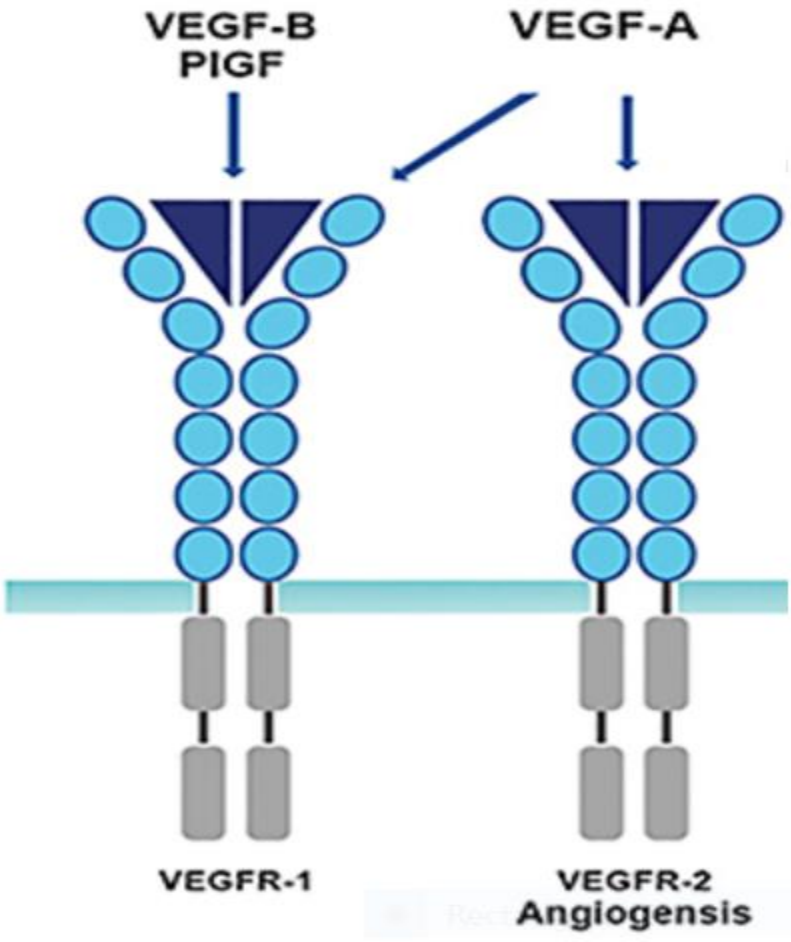


Figure 5: VEGF Receptors 1 and 2 (Reference: Sharma A, Bandello F, Kuppermann B, Makam D, Research Efforts are going beyond targeting VEGF)

1.13.4 Platelet Endothelial Cell Adhesion Molecule-1(PECAM-1)/CD31

PCAM-1 is a transmembrane glycoprotein located on the blood endothelial cells, platelets and on leukocytes. It has been shown to play a major role in angiogenesis. It facilitates leukocyte movement through endothelial intercellular junction during inflammation. [34-36]

1.14 Innovation

Angiogenesis play a major role in functional bone regeneration. The current gold standard protocol for the repair of critical size defects is the standard autografts.

Due to limitations associated with autografts, and allografts, stem cell-based tissue engineering emerged as a viable approach to regenerate biological tissue substitute for critical bony defects. [4,7-11] Mesenchymal stem cells (MSCs) from different origins (For example; bone marrow, adipose tissue cells, urine) have been shown to differentiate to endothelial cells following supplementation of vascular endothelial growth factor (VEGF) in media.[7-9,11] Limited studies have addressed on orofacial tissue derived mesenchymal stem cells. It has been shown that deriving stem cells from Orofacial tissues are minimally invasive and easily accessible. The origin of GMSCs is from neural crest cells and they differentiate into cartilage and bone to form craniofacial skeleton.[4] Although, a technological development is required to regenerate a highly vascularized and functional bone in craniofacial region, to the best of our knowledge, none of the study has ever addressed such a technique to differentiate GMSCs to endothelial cells in the regenerated bone tissue. In this project, for the first time, we propose to differentiate gingival stem cells to endothelial progenitor cells by supplementing with VEGF. This project will be innovative in terms of developing a highly vascularized and vital bone in the craniofacial region by differentiating GMSCs into endothelial cells. It will eliminate drawbacks associated with co-culturing (that is, co-culture of endothelial and osteogenic cells) technique. And also, it would be possible to use gingival stem cells as the single cell source to enhance angiogenesis during bone regeneration.

1.15 Objectives

The long-term goal of this project is to develop *in-vitro* pre-vascularized bone tissue constructs which will be transplanted to the large defect for regeneration of bone with sufficiently functional vascular networks. Thus, the translational significance of this project represents the novel tissue engineering approach to regenerate a highly vascularized and vital bone during bone regeneration in the craniofacial region.

1.16 Specific Aim

The aim of this study is to examine the effects of VEGF on induction of *in vitro* differentiation of gingival stem cells into endothelial cells.

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

Commercially available Recombinant Human VEGF, PECAM antibody were purchased from R&D Systems (Minneapolis, MN) was used for the study. Human Umbilical Vein Endothelial cells and were obtained from Lonza, (Allendale, NJ). Primers used for quantitative PCR were obtained from Life Technologies. All other required lab supplies were acquired from Sigma (St. Louis, MO).

2.2 Overall Study Design

The methods for isolation of gingival stem cells from human gingiva tissue has already been established in our laboratory. The gingival tissue was obtained from the NSU clinics following the guidelines of Institutional Review Board (IRB). In this study, cryopreserved GMSCs were revived and the cells from 5 donors from male and female have been used for all experiments. Cells were cultured under standard culture conditions. Cells that have reached 70-80 % confluency were induced with different concentrations of angiogenic medium (Dulbecco's modified Eagle Medium (DMEM) + 10% fetal bovine serum (FBS) + 1% antibiotics + VEGF 10, 50, 100 ng/mL) for one week. Human Umbilical Vein Endothelial cells (HUVECs) were used as a positive control. The endothelial cell differentiation was measured by quantitative PCR, immunofluorescence, tube formation assays. In addition to that, scratch assay was performed to determine the migration of endothelial cells. (Figure 6)

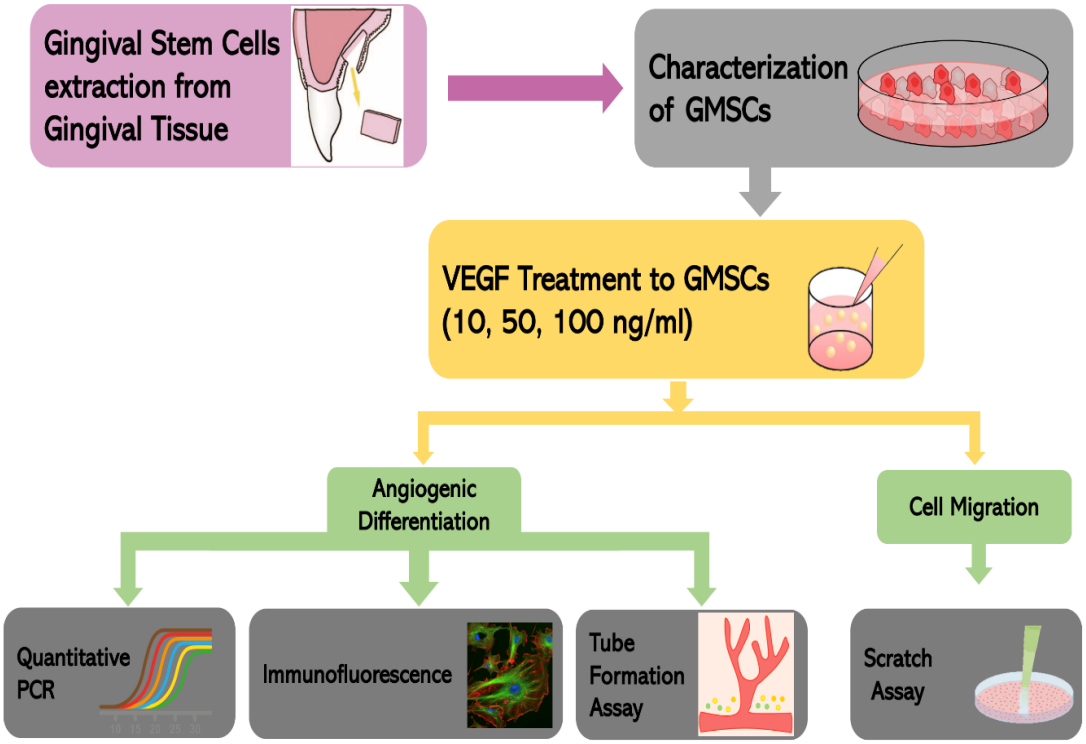


Figure 6: Overall Methodology Design

2.3 Cell Culture and Characterization of GMSCs

The GMSCs obtained from human gingival tissues were cultured in 75cm² culture flasks in growth medium (DMEM) supplemented with 10% FBS and 1% antibiotic) and were incubated at 37°C in 5% CO2 atmosphere. After 72 hours of culture, non-adherent cells were removed.[37] The growth medium was changed every 2 days. The cells obtained from passage 2 were subjected to Flow-cytometric analysis to confirm positive to mesenchymal markers CD44, CD73, CD90, and CD105 and negative to CD34 (hematopoietic stem cells).[20] These specific markers were measured at the facilities at University of Miami using a fluorescent activated cell sorter FACAria IIIu (BD Biosciences, San Jose, CA) with adjusted florescence compensation setting.

2.4 VEGF Treatment to GMSCs

A recombinant human VEGF protein (Source: *Spodoptera frugiperda*, *Sf* 21 (baculovirus) - derived Ala27-Arg191) obtained from R n D systems, Minneapolis, MN (catalogue # 293-VE-050) with a purity of >97%, with endotoxin level <0.01 EU per 1 µg was used for this study.

Gingival stem cells at 70- 80% confluency were treated with angiogenic differentiation medium (DMEM, 10% fetal bovine serum, 1% antibiotic supplemented with different concentrations of VEGF-10, 50, 100 ng/ ml) for 7 days. Medium was changed every 2 days.[10] The cells without VEGF treatment, and HUVECs were designated as negative and positive control groups respectively (Table 1). Cell morphology was monitored after induction with VEGF. Table 1 represents control and experimental groups studied for the study.

Control Groups	**Experimental Group**
Human Umbilical vein endothelial cells (HUVECs) - Positive control	10 ng/ml VEGF treated Gingival Stem Cells
Undifferentiated Gingival stem Cells - Negative control	50 ng/ml VEGF treated Gingival Stem Cells
	100 ng/ml VEGF treated Gingival Stem Cells

Table1: Control and Experimental Groups with HUVECs as positive control

2.5 Scratch Assay

This assay typically involves culturing a confluent cell monolayer and then displacing a group of cells by creating a scratch through the monolayer. In a 12 well plate $1x10^5$ per well GMSCs were seeded and these were maintained at 37 °C and 5% CO_2 for 24 h to permit cell adhesion and the formation of a confluent monolayer. These confluent monolayers were then scored with a 1ml sterile pipette tip to leave a scratch of approximately 0.4–0.5 mm in width. Culture medium was then immediately removed (along with any dislodged cells). The removed medium was replaced with a fresh medium which served as control group. 50ng/ml VEGF was supplemented to the experimental group. The cell migration was monitored at 1,4 and 17 hours. The cell migration was then analyzed microscopically by capturing images at the beginning and at the regular intervals to see the endothelial cell migration for closing the scratch.[38] [39] (Figure 7)

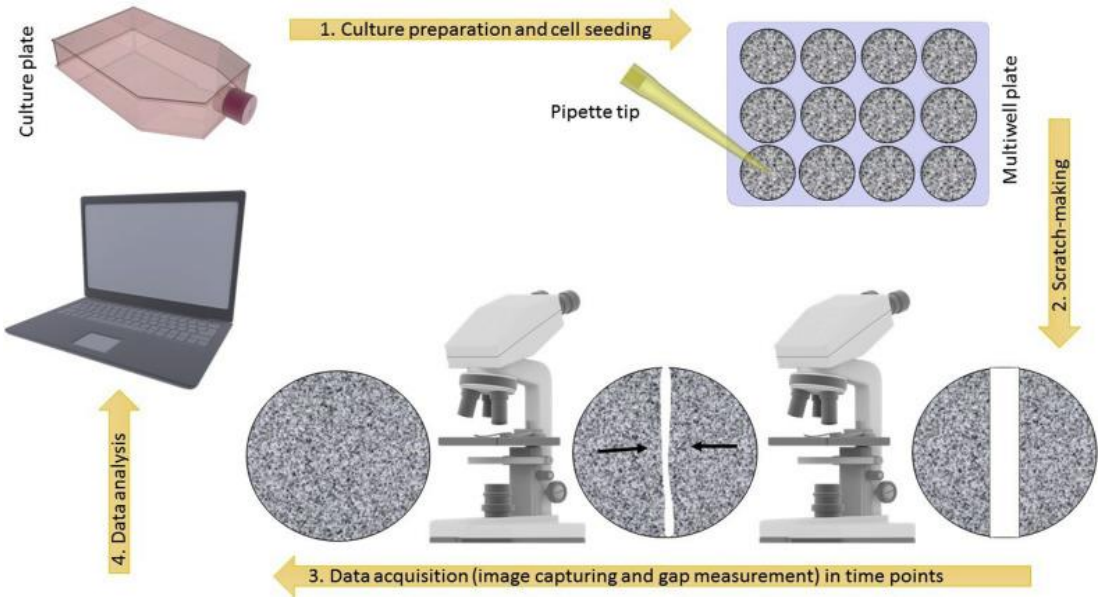


Figure 7: Scratch Assay: The technique involves basic steps 1) cell seeding and preparation; 2) making a linear thin scratch "wound" (creating a gap) 3) data acquisition through microscopic image capturing and gap measurement at each time point; and 4) data analysis. (Reference: Grada A, Otero-Vinas M, Prieto-Castrillo F, Obagi Z, Falanga V. Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay)

2.6 Gene Expression by Quantitative PCR

Cells differentiated for 7 days were harvested and lysed in Trizol. The RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) extraction method established in our laboratory and quantified. CDNA was prepared using high capacity reverse transcription kit (life Science Technologies, Carlsbad, CA) according to manufacturer's recommendations.[40] The expression of endothelial marker genes VEGF receptors VCAM-1, KDR, PCDH12, FLT-1, and PECAM-1 were measured using qPCR (Figure 8). The primers used are listed in Table 2.

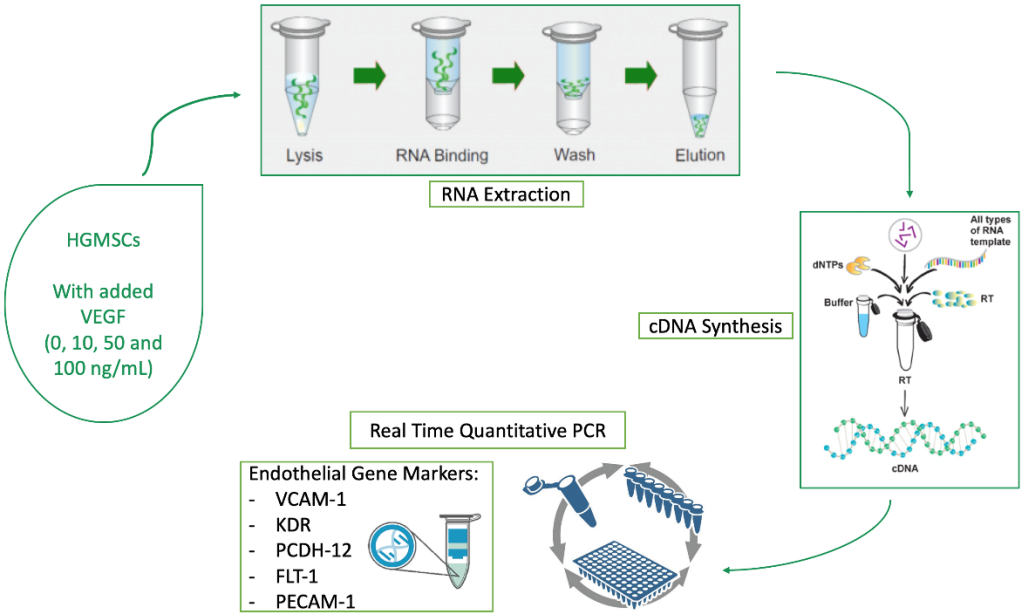


Figure 8: Quantitative PCR Gene Expression Studies

Serial #	Gene	Gene expression assay used
1	PECAM-1	Hs01065279
2	VCAM-1	Hs01003372
3	KDR	Hs00176676
4	FLT-1	Hs01052961
5	PCDH12	Hs01007986
6	B-Actin	Hs00194899

Table 2: Primers used in Quantitative PCR

2.7 Immunofluorescence

The GMSCs treated with or without 50ng/ml VEGF were cultured for 7 days and subsequently fixed with 4% pre-chilled (4°C) paraformaldehyde. The cells were incubated at room temp for 15 mins and were rinsed 3 times with ice cold PBS for

5 mins each. The cells were incubated with 2 % BSA, 22.52 mg/ mL glycine in PBST (PBS + 0.1 % Tween 20) for 60 mins to block unspecific binding of the antibodies. After that, diluted primary antibody (PECAM-1) was added into the cells in each well containing 1% BSA in PBST and kept for overnight incubation in 4º C. Cells were washed with PBS 3 times for 5 mins each followed by incubating with secondary antibody (Goat Anti-mouse IgG Alexa Fluoro-Plus™ 488). After incubation, the cells were washed with PBS. DAPI (life Technologies, Carlsbad, CA) was used as counter stain. The cells were then observed under the fluorescence microscope.[10]

2.8 Tube Formation Assay

The HGMSCs were treated with 0, 10, 50 and 100 ng/ml VEGF for 7 days. 50 µL of Matrigel (mixture of extracellular matrixes; laminin, nidogen, collagen and heparan sulfate proteoglycans, Corning) was added to each of the 16 wells in a 96-well plate and incubated for 1 hour at 37°C to solidify. Cells were then trypsinized and suspended. The suspended cells were seeded on top of the Matrigel. After incubation for 0,1, 3, 6 and 12 hours, the capillary like structures were examined under the microscope and pictures were captured using Cellsens Standard Software.[10] (Figure 9)

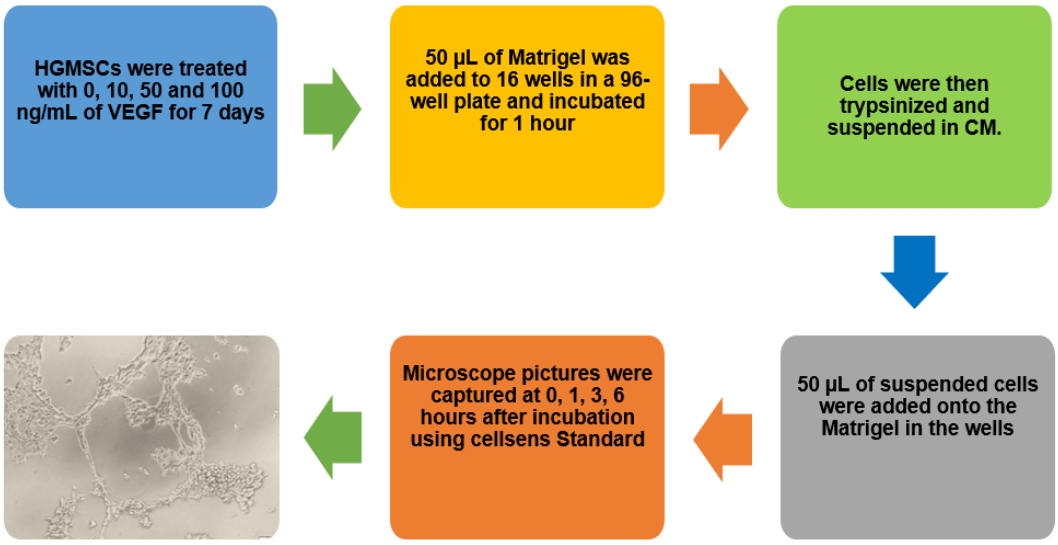


Figure 9: Tube Formation Assay

2.9 Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 5.0software (GraphPad Software, La Jolla, CA, USA). Data presented in this study are means ± standard deviation of the mean. For quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), relative gene expression ratios were determined using the 2Δct mathematical model. Samples were analyzed at least in five independent experiments (n=5) using triplicates at minimum for quantitative PCR. For all other experiments three independent experiments (n=3) were conducted. To evaluate differences between or among groups (control group and experimental group), analysis of variance (ANOVA) was performed. In order for statistical tests to be significant, a P-value < 0.05 is selected.

CHAPTER 3 RESULTS

3.1 Isolation and Culture of Human Gingival Stem Cells

The isolated cells from the tissue were plated at a density of 2 x 10^4 cells/cm^2 and incubated with growth medium (DMEM supplemented with 10% FBS and 1% antibiotics). The cells became 70 to 80% confluent after 3-5 days. The cells showed homogenous, tightly adherent, spindle-shaped and fibroblast like morphology.

3.2 Flow Cytometry Analysis

The cells obtained from passage 2 were subjected to flow cytometry. The flow cytometry confirmed expression of surface markers CD105, CD90, CD73 (all above 90%) and lack the expression of hematopoietic stem cell marker CD34. (Figure 10)

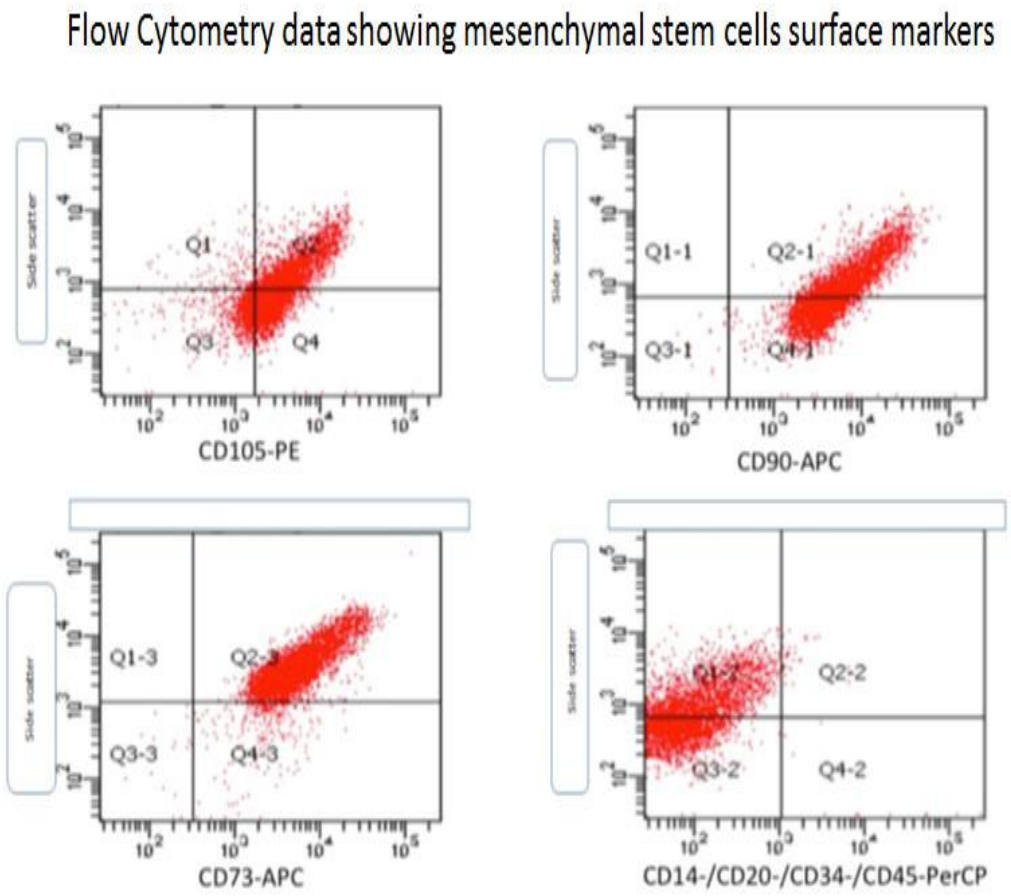


Figure 10: Flow Cytometry data showing surface markers of Mesenchymal Stem Cells. The cells showed over 90% positive to CD73, CD90, CD105 and negative to CD34

3.3 Morphology of Cells after Induction of Angiogenic Medium

Cell morphology after VEGF treatment was monitored under light microscope. Under phase contrast view, plated cells show spindle shaped structures at day 3 of induction with VEGF (Figure 11B). The cells shape is not exactly like that of typical endothelial cells, so called cobble-stone appearance. Majority of cells still showed fibroblast-like structure on the 7th day of differentiation. (Figure 11C)

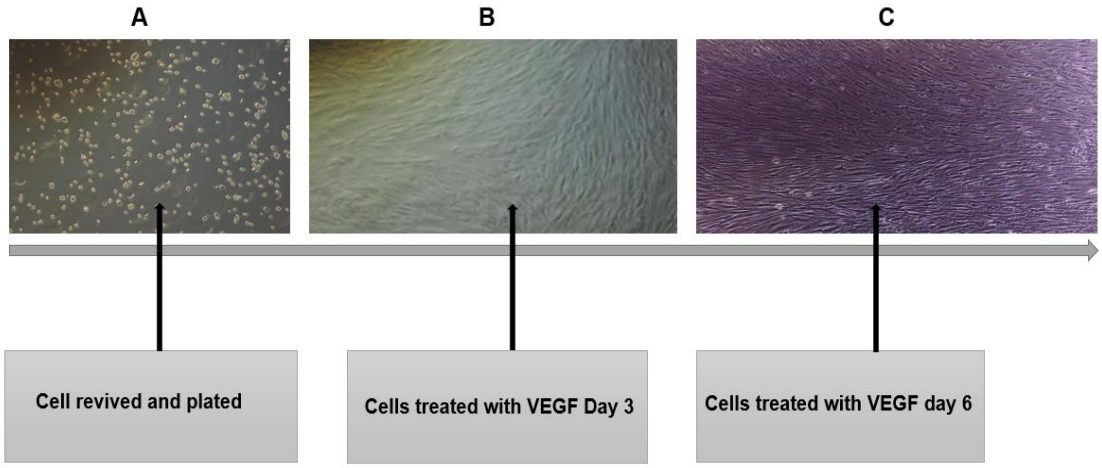


Figure 11: Morphology of cells after plating and after VEGF treatment on day 3 and 6

3.4 Scratch Assay

In- vitro Scratch assay was conducted to track the migration of endothelial cells. The images were captured at the beginning, 4 hours and at 17 hours. Results showed significant endothelial cell migration towards the scratch as compared to control group (Undifferentiated GMSCs with CM only). At 0 hour, cells in experimental group just started migrating. We observed more migration of cells in experimental groups at 4 hours. At 17 hours, cells migration in VEGF induced cells showed more than 90 % closure when compared to control (Figure 12).

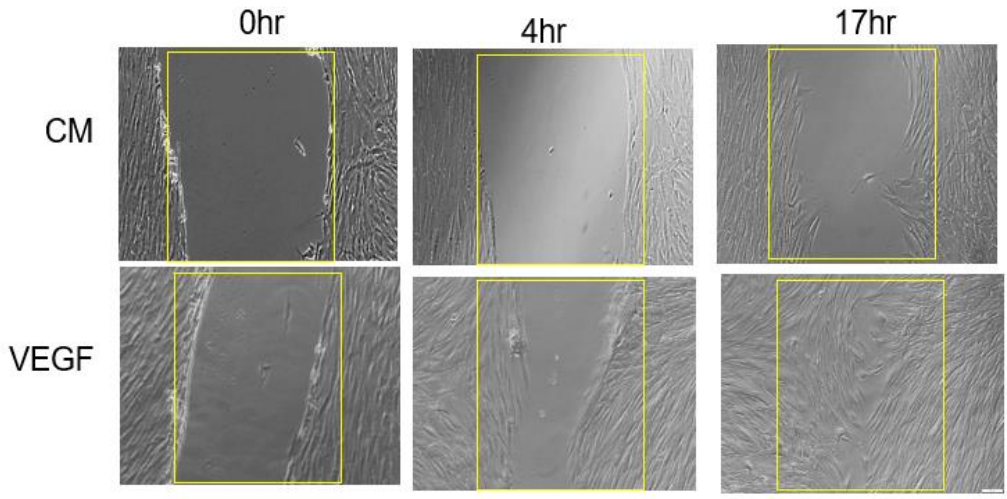


Figure 12: Scratch assay: Control group (CM) showed much slower migration of cells. 50 ng/ml VEGF induced GMSCs showed more than 90% closure at 17 hours.

3.5 Gene Expression Studies of Various Endothelial Markers of GMSCs

The expression of endothelial markers genes VEGF receptors (KDR, FLT-1), PCDH12 VCAM-1, PECAM-1 were measured at day 7 post VEGF treatment. The VEGF enhanced the gene expression of all endothelial markers in a dose dependent manner. While VCAM-1 and KDR expression was significantly upregulated at all concentrations (P= 0.0041, P= 0.003) respectively, the upregulation of PCDH12 (P< 0.0001), FLT1 (P< 0.0001) and PECAM-1 (P< 0.03) was maximum at the cells treated with 50ng/ml VEGF. VCAM-1 expression significantly increased 75 % at 10, 50 ng/ml and almost 100% increase at 100 ng/ml (Figure 13). While KDR expression increased by significant 50 % and 75 % at 50 and 100 ng/ml respectively, there was no significant increase in the cells treated with 10ng/ml compared to control (Figure 14). For PCDH12, the dose dependent increase has been observed (Figure 15). As shown in Figure 16, for FLT-1, there was 25 % and 50 % increase at 10 and 100 ng/ml respectively, and expression

almost doubled at 50 ng/ml. Furthermore, PECAM-1 showed significant increase of 50 % over the control at 50 ng/ml concentration (Figure 17). Overall, the results demonstrated a dose dependent increase of endothelial markers. However, there was no significant difference between 50ng/ml and 100ng/ml in any of the gene expression. The relative gene expression of VCAM-1 has significantly increased in GMSCs, however, the expression of all other genes was not comparable to HUVEC cells (Data not shown).

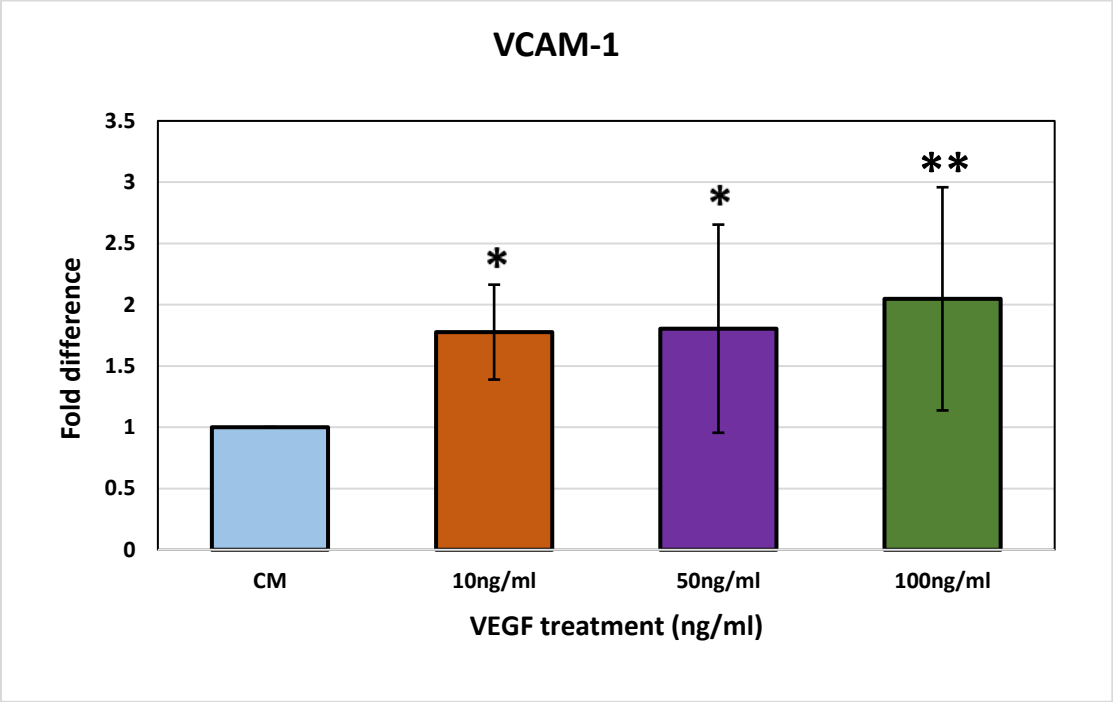


Figure 13: GMSCs: Relative Gene Expression of VCAM-1 were analyzed 5 independent times on 5 different donors, leading to p-value = 0.0041

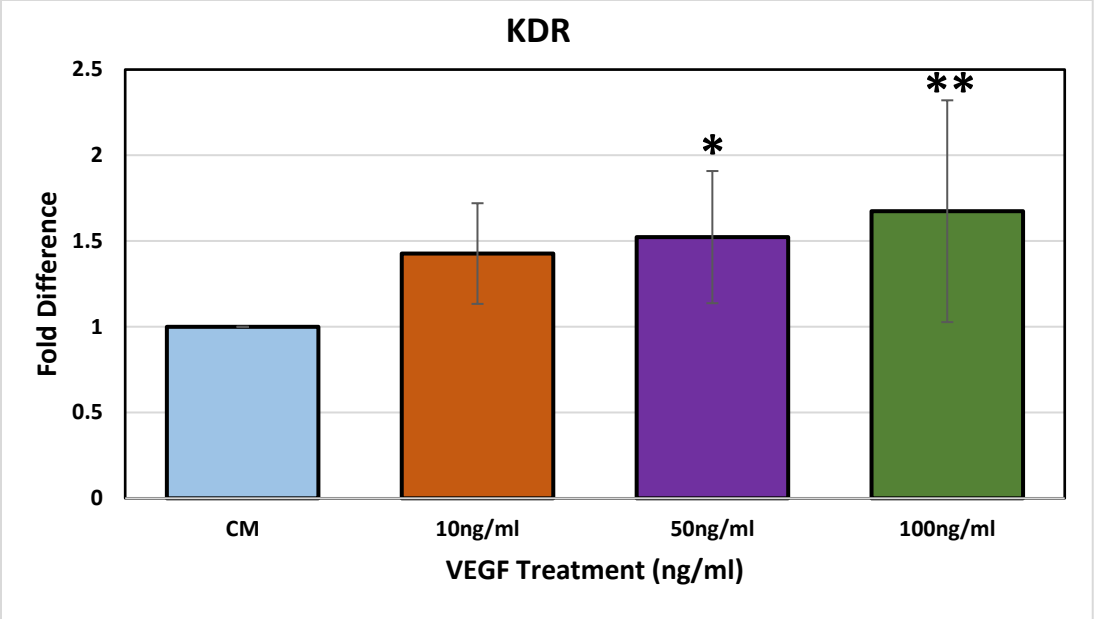


Figure 14: GMSCs: Relative Gene Expression of KDR were analyzed 5 independent times on 5 different donors, leading to p-value =0.003

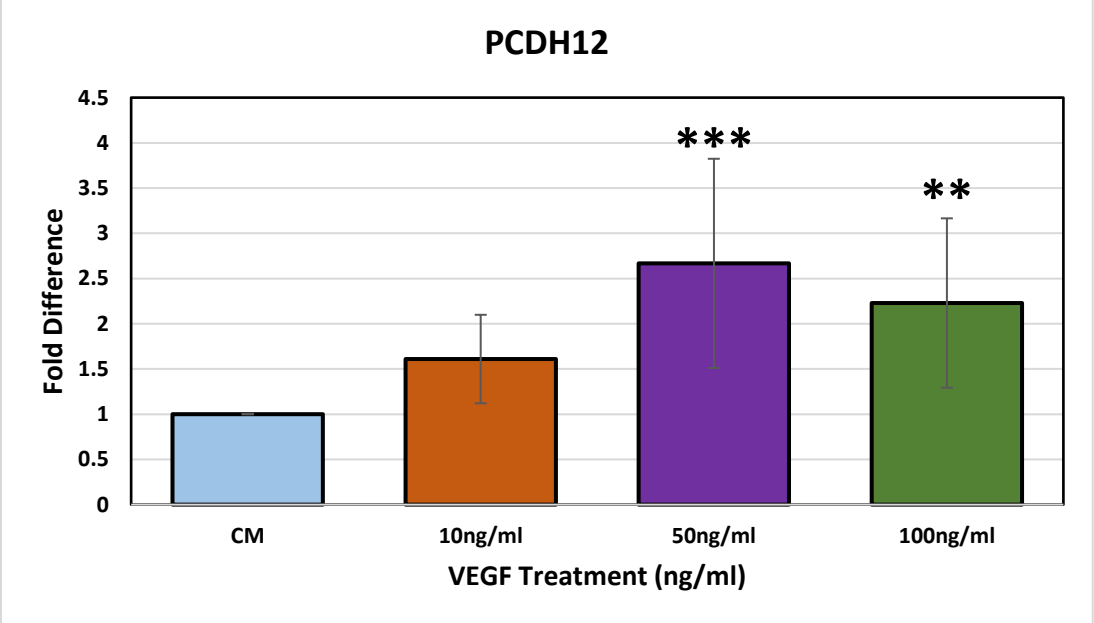


Figure 15: GMSCs: Relative Gene Expression of PCDH12 were analyzed 5 independent times on 5 different donors, leading to p-value <0.0001

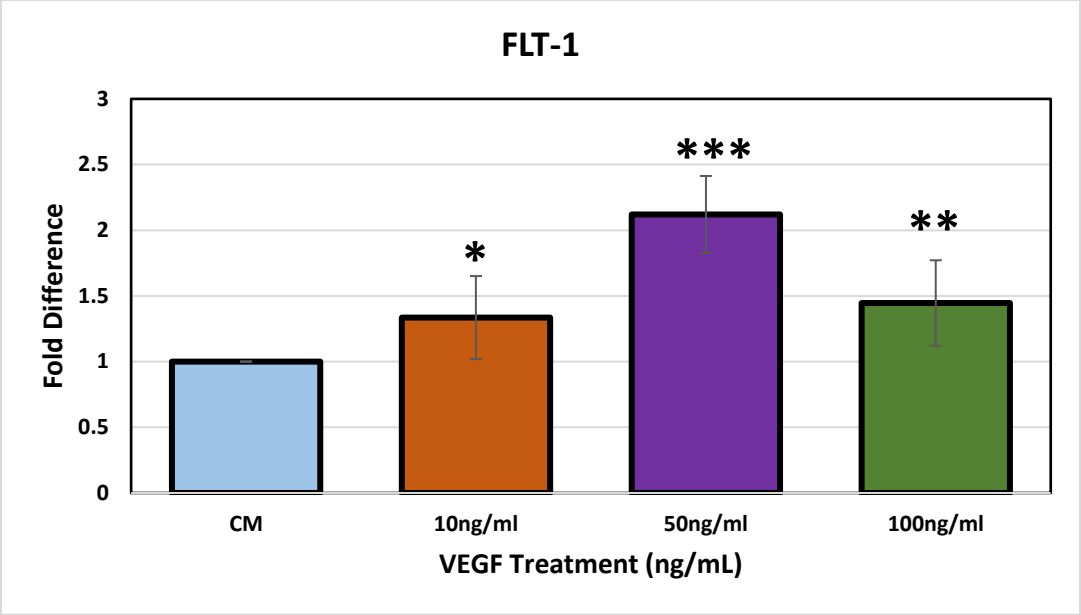


Figure 16: GMSCs: Relative Gene Expression of FLT-1 were analyzed 5 independent times on 5 different donors, leading to p-value <0.0001

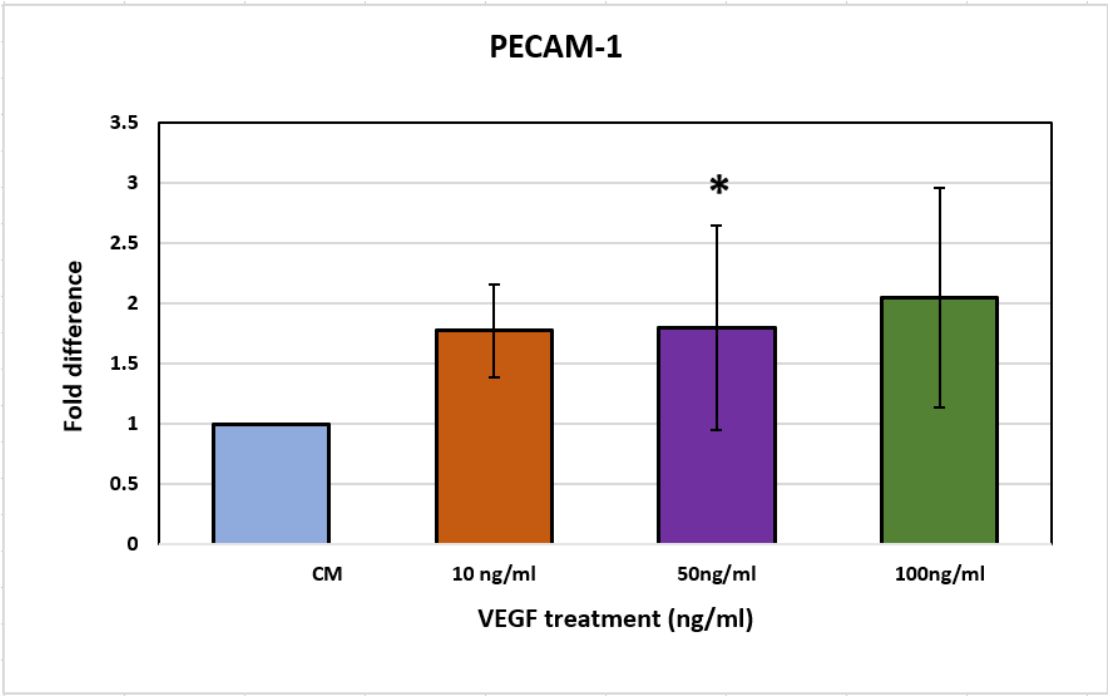


Figure 17: GMSCs: Relative Gene Expression of PECAM-1 were analyzed 5 independent times on 5 different donors, leading to p-value <0.03

3.6 Immunofluorescence Assay

Both HUVECs and GMSCs were treated with 50ng/ml of VEGF for one week. After 7 days, immunofluorescence assay performed to measure the expression of PECAM-1. Immunofluorescence analysis demonstrated the expression of PECAM-1 in differentiating GMSCs. Furthermore, results showed expression of PECAM-1 in GMSCs is comparable with the expression in HUVECs. (Figure 18)

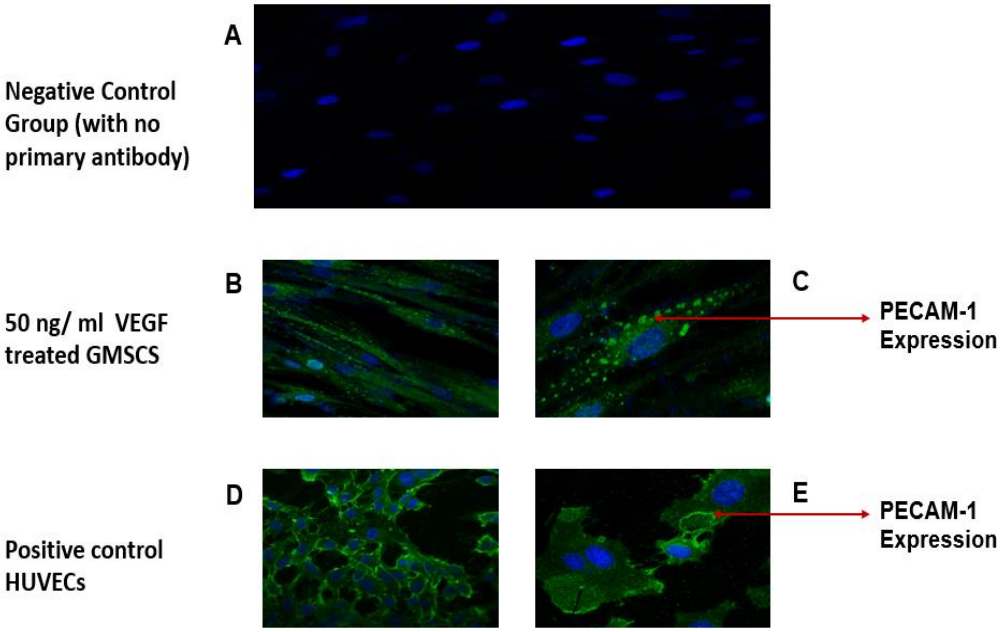


Figure 18: Immunofluorescence assay: Expression of PECAM-1 was observed in the cell membranes of both GMSCs with 50 ng/ ml VEGF treatment and HUVECs. (Figures B, D). Figure C and E showed magnified picture of cell for both VEGF induced gingival stem cells and positive control. Negative control did not express PECAM-1 (Figure A)

3.7 Tube Formation Assay

The functionality of the differentiating GMSCs angiogenic potential was assessed in tube-forming assays, demonstrating that seeding of 3x103 cells on Matrigel started forming tubes from 1 hour and it continued until 6 hours.

While the experimental group was the GMSCs induced with 10, 50 and 100 ng/ml of VEGF for 7 days, the undifferentiated GMSCS (Cells treated with complete medium at 0 ng/ml VEGF) served as control group. The functional behavior of the differentiated cells showed more capillary like structures. Tube formation was compared at 0, 1, 3 and 6 hours. At 0 hour, cells were just plated and all concentrations look similar (Figure 19). At 1 hour, cells started organizing slightly for tube formation in both control and experimental group. However, that was prominent in the cells treated with 10 ng/ml and 50ng/ml (Figure 20). After 3 hours, in 10 and 50 ng/ml, the cell clusters showed branching and tight interconnections, while few capillaries were detected at 100 ng/ml concentration and no capillaries formation was seen in control group (Figure 21). 6 hours afterwards, tube formation was greatly enhanced at all concentrations. We observed increased density of cell clusters at 10, 50 ng/ ml concentration. Tube formation interconnection was also noticed in undifferentiated cells with CM only. At 100 ng/ ml, short tube formation was observed and we noticed initiation of disintegration of capillaries (Figure 22). After 12 hours, capillaries fully disintegrated and no more tube formation was visible. (Figure 23)

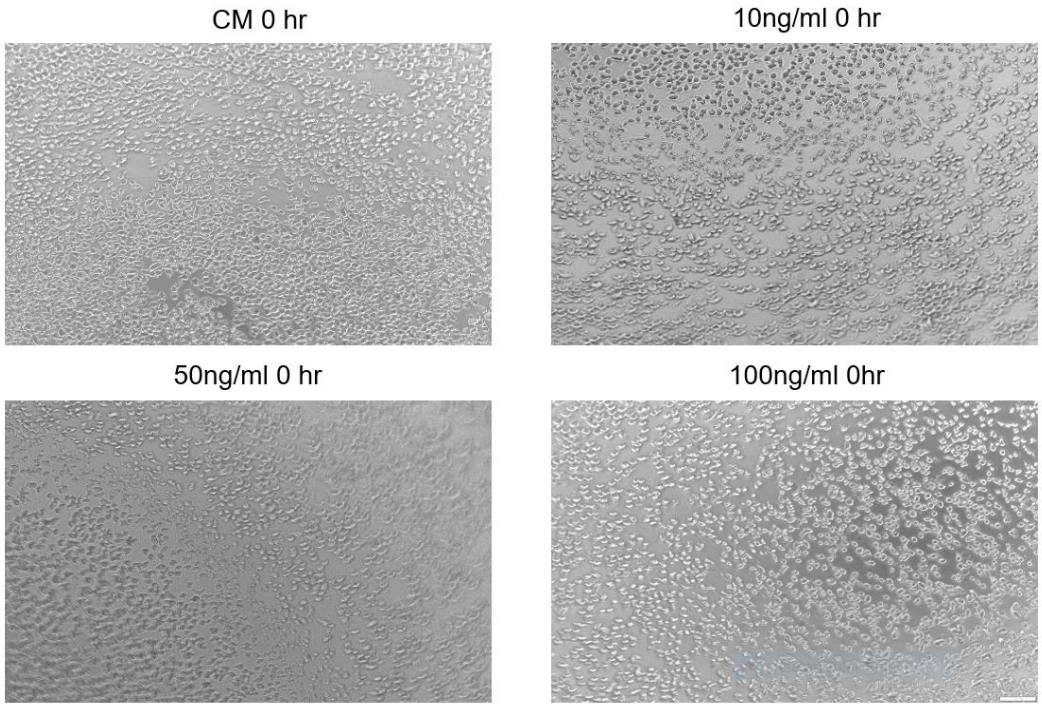


Figure 19: Tube formation assay at 0 hour: Cells just plated onto the Matrigel

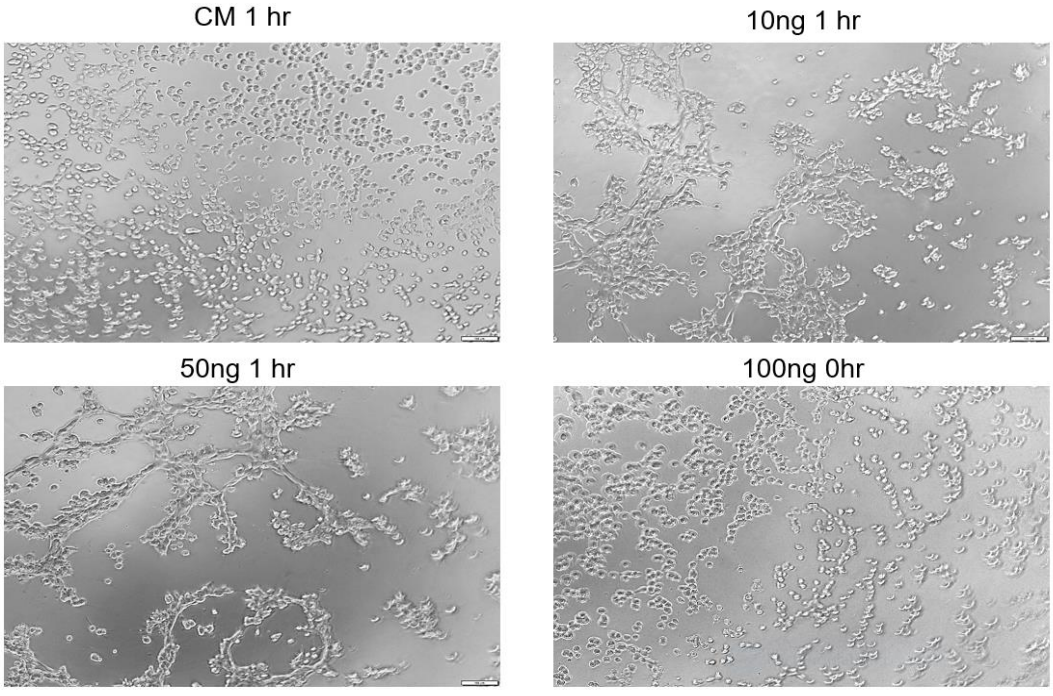


Figure 20: Tube formation assay at 1 hour: Cells started organizing for tube formation, however this was more prominent in cells at 10 and 50 ng/ml

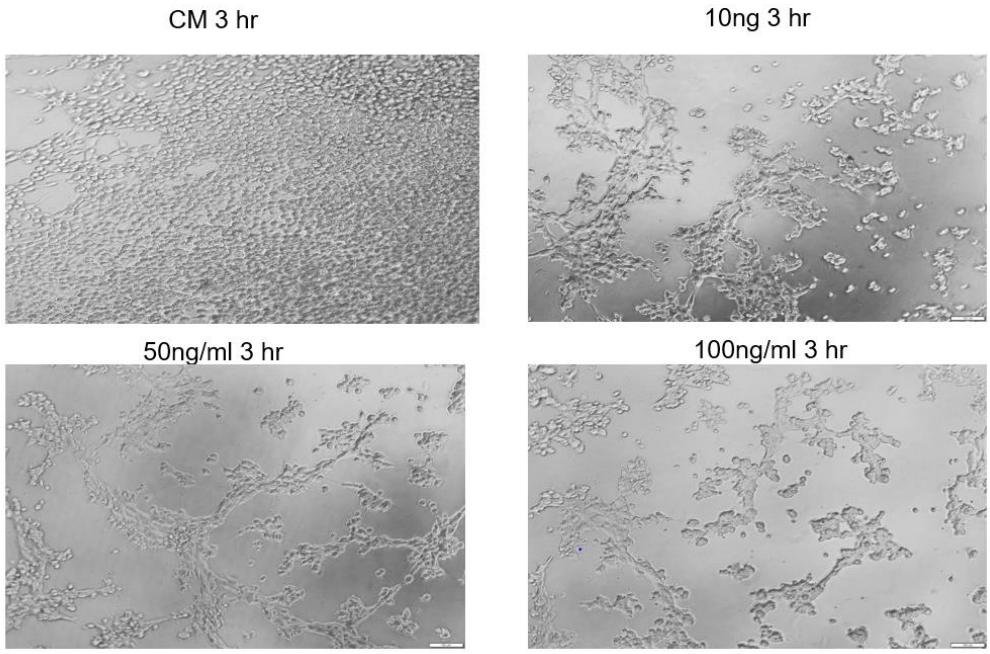


Figure 21: Tube formation assay at 3 hours: Cells started showing branching and interconnections at 10 and 50 ng/ml, while few capillaries were detected at 100 ng/ml concentration. No capillaries formation was seen in undifferentiated GMSCs at 0 ng/ml concentration (CM)

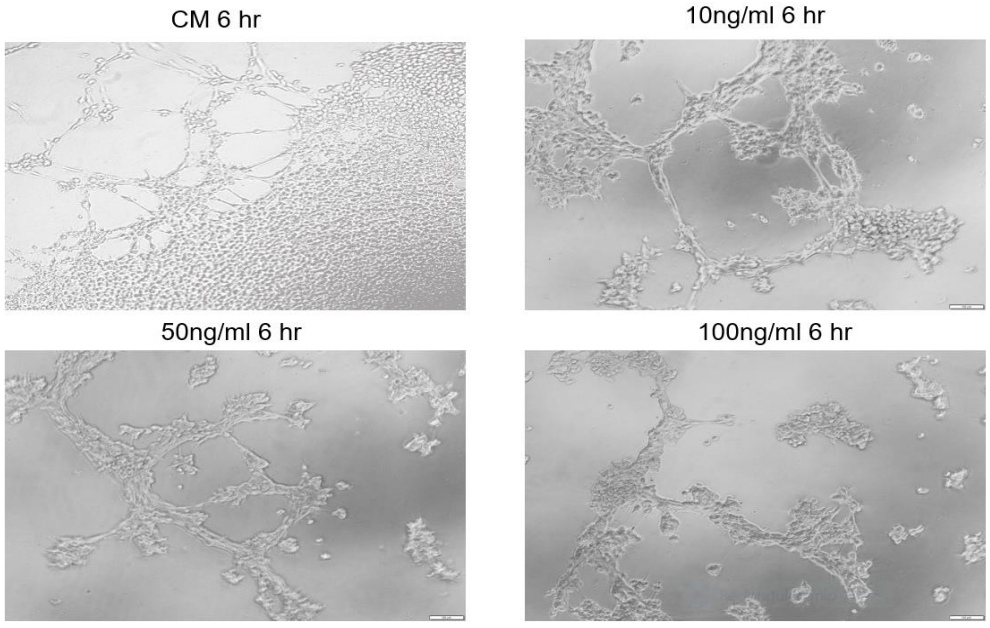


Figure 22: Tube formation assay at 6 hours: Tube formation was greatly enhanced

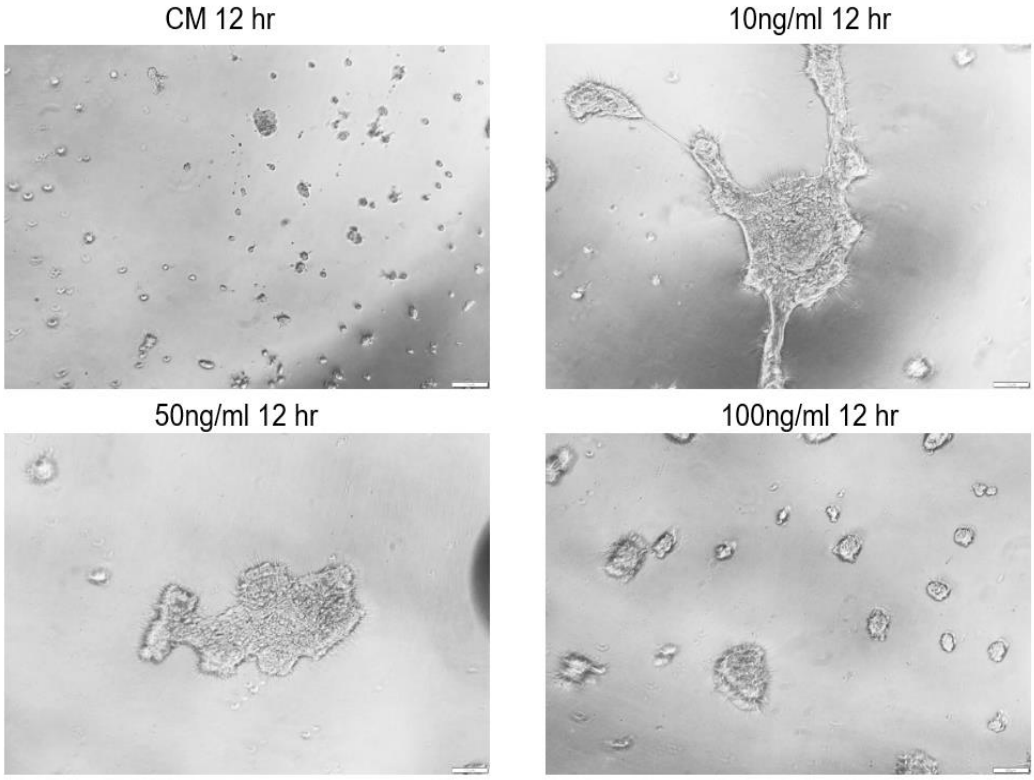


Figure 23: Tube formation assay at 12 hours: Capillaries disintegrated and no more tube formation was visible

CHAPTER 4 DISCUSSION AND CONCLUSIONS

4.1 Discussion

Mesenchymal stem cells (MSCs) are of great interest in regenerative medicine as they have promising potential for clinical use. MSCs are pluripotent progenitors that can differentiate into a variety of cell types while maintaining the self-renewal property. Their applications have been extensively studied in bone tissue engineering. [4,7-11] However, vascularization remains a challenge in bone tissue engineering. Vasculature is essential for bone formation and bone remodeling during fracture healing. Studies have shown that endothelial cells can enhance bone regeneration ability.[7] Mature endothelial cells have been used for vascularization in bone tissue engineering, but these have limited proliferative capacity and cells are not sufficient for repair and reconstruction of blood vessels during bone regeneration.[12] Sufficient evidences in the past indicate that mesenchymal stem cells *in- vitro* have the potential to differentiate into endothelial progenitor cells.[9,10,41] Transdifferentiation of MSCs to endothelial cells have many advantages as serve as autologous stem cell source with highly proliferative with self-renewing capability. Differentiating MSCs to endothelial cells have added advantage, as the endothelial progenitor cells are more proliferative than differentiated endothelial cells.[12] MSCs differentiating into endothelial cells have been reported in bone marrow derived stem cells, adipose derived stem cells and stem cells derived from Wharton's jelly.[7,10,12,41,42] The angiogenic differentiation of dental pulp derived stem cells have been reported recently.[43] Indeed, the stem cells derived from dental tissue secrete angiogenic factors to stabilize vascular

networks.[43,44] VEGF has pivotal role on endothelial cell proliferation, migration and initiating and regulating angiogenesis.[6,13] Neovascularization induced by VEGF represents an appealing approach for bone tissue engineering. Many scientists explored the differentiation of MSCs utilizing VEGF. For example, Oswald et al. reported the differentiation of human bone marrow -derived mesenchymal stem cells to endothelial progenitor cells with VEGF activation.[10] Similarly, Cao et al. have demonstrated that the human adipose tissue-derived stem cells differentiated into endothelial progenitor cells *in-vitro* with VEGF and b-FGF activation.[9] These evidences indicated that VEGF can support the differentiate mesenchymal stem cells into endothelial progenitor cells (EPC). EPC have a higher survival rate and 10 times more proliferative as compared to mature endothelial cells.[7,12] In this study, we have investigated the effect of vascular endothelial growth factor (VEGF) to induce the differentiation of GMSCs into endothelial lineage.

Our approach is to utilize the minimal dose of VEGF to induce the angiogenic differentiation in GMSCs. In this study, we used different concentrations of VEGF (10, 50, 100 ng/ ml) were tested for its ability to induce endothelial differentiation from GMSCs *in vitro*. After 7 days of VEGF treatment with different concentrations, gene expression of angiogenic marker genes was measured using quantitative PCR. To confirm the results immunofluorescence and tube formation assay was conducted. The overall results indicated that VEGF induced stem cell differentiation into endothelial like cells. The angiogenic differentiation medium that

we have used consists of DMEM with 10% Fetal Bovine Serum (FBS) and 1% antibiotics supplemented with VEGF. Table 3 gives the details of culture conditions of various researchers used.

Author and Year	Type of MSCs used	Angiogenic Culture Medium	How many days of Differentiation
Khaki, 2018	Mesenchymal stem cells (MSCs)	VEGF 50 ng/ml	10 days
Cao, 2005	Human adipose tissue-derived stem cells	VEGF 50 ng/ml and b-FGF 10 ng/ml	2-3 days
Wang, 2018	Bone marrow mesenchymal stem cells	VEGF, basic fibroblast growth factor (bFGF), insulin like growth factor (IGF), epidermal growth factor (EGF)	14 days
Aksel, 2017	Primary human DPSCs or swine DPSCs	Endothelial growth medium (EGM-2)	7 Days
Bento, 2013	Human Stem cells from Exfoliated Deciduous Teeth (SHED)	Endothelial cell growth medium (EGM-2MV) supplemented with 50 ng/mL VEGF	28 days
Oswald, 2004	Human bone marrow derived mesenchymal stem cells	50 ng/ ml VEGF	7 days

Table 3: Culture Medium used in various studies

For example, Oswald et al used 2% FBS in the culture medium supplemented with VEGF,[10] however, in our study the cells we used 10% FBS in culture medium, as our pilot study conducted with 2% and 5% FBS in culture media demonstrated that the cells could not survive in low concentrations of FBS (either 2% or 5%). In our study, we have initially used various concentrations of VEGF for gene expression studies and tube formation assay. Our data revealed that 50ng/ml was the optimal concentration at which the maximal upregulation of marker genes and vessel formation observed. Hence, we used 50ng/ml for all other experiments.

Cell migration is the coordinated movement of a group of cells that maintain the inter cellular connections and collective polarity. Cell migration studies are hallmark in angiogenesis, would healing and cancer invasion. It can be studied by a variety of methods; one of the popular methods, which is very often used, is the scratch assay or wound healing assay.[38] Our studies indicated that VEGF enhanced the cell migration compared to untreated cells. Our data is in agreement with the studies of Fiedler et al.[45] They demonstrated that VEGF participates induce the migration of bone marrow derived progenitor cells. Cell migration is a key understanding process in wound healing.[46] Thus our studies indicated that VEGF has a potential role inducing cell migration and wound healing process.[45,46] VEGF is a key regulator of angiogenesis through cell migration and proliferation of vascular endothelial cells.[47] Many other researchers also demonstrated that VEGF has a significant role in the cell migration.[45,46] Our data showed that the cells started migrating in VEGF treated cells and the migration rate increased over time compared to control (cells treated with CM). The VEGF treated cells migrated and almost 90 % closed the scratch 17 hours. A similar pattern was observed in several earlier studies.[38,39,45,46]

The results of quantitative PCR demonstrated that the endothelial markers VCAM-1, PECAM-1, KDR, FLT-1, PCDH12 in GMSCs induced with VEGF for 7 days significantly upregulated compared to undifferentiated cells. When compared with HUVEC cells, the VCAM-1 expression is strikingly significantly upregulated than the expression in HUVEC cells. However, the expression of other genes was significantly less than HUVEC cells grown in similar culture conditions. A similar

pattern of down regulation was reported earlier, suggesting that a tissue specific hierarchical pattern of gene expression.[41] Duration of differentiation and the culture medium used for angiogenic medium might be contributing factors. While differentiation of Wharton's Jelly derived stem cells cultured in M199 medium occurred in 4 days, in SHED cultured in VEGF induced differentiation medium have not expressed endothelial markers until 28 days.[41,48] In dental pulp derived stem cells (DPSC) cultured in EGM medium, and bone marrow stem cells cultured in VEGF, basic fibroblast growth factor (bFGF), insulin like growth factor (IGF), and epidermal growth factor (EGF) expressed angiogenic markers in 7 days and 14 days respectively.[43,49] From these studies it is evident that culture medium and duration of differentiation contribute important factors.

Angiogenesis is a tightly organized sequence of cellular events characterized including endothelial cell migration, invasion and differentiation into capillaries.[42] *In vitro* endothelial tube formation assays are used as a model for studying endothelial differentiation. This assay is employed to determine the ability of various compounds such as growth factors or drugs to promote or inhibit the tube formation. Essentially, this assay measures the ability of cells plated at sub confluent densities with appropriate extracellular matrix support to form capillary like structures.[42,49] Our data on tube formation assay revealed that VEGF induced vascular like network in classical tube formation assay. GMSCs induced with VEGF at 10 and 50 ng/ml formed the tubes with maximum at 6 hours. After 8 hours, the cells started clumping, and we could observe large clumps at 12-hour time. Our studies revealed that after one hour they started migrating towards the

center and start organizing tubule. A dose dependent trend has been seen in our study. The tubes formed with dense branching at 10ng and 50 ng/ml. In 50 ng/ml the tube density was maximum.

4.2 Conclusions

The regeneration of defect and lost tissue in oral region is the goal of our stem cell research. For the first time, our study demonstrated that GMSCS were able to differentiate into endothelial like cells. The advantage of utilizing the GMSCs isolated from gingiva tissue is that, they are obtained as a discarded tissue in the clinics during flap surgery procedures or third molar extractions. Furthermore, they can serve as autologous stem cell source with robust proliferation rate and minimal risk of immune-rejection. In addition to their neural crest origin, the high self-renewal capacity, wound healing ability and their plasticity makes GMSCs suitable for bone tissue engineering applications. On the contrary, the stem cells developed from bone marrow cells require the invasive surgery. Additionally, the endothelial differentiation ability of GMSCs, will expand their scope for many other tissue engineering applications including bone and cardiac tissue engineering applications. Thus, our study addressed, in part, the existing challenges to develop a vascularized tissue engineered bone. Further, in vitro and in vivo studies are warranted to gain more insights on the effects of VEGF as enhancer of angiogenic/osteogenic coupling in stem cell based craniofacial bone regeneration.

5. RAW DATA

	A	B	C	D	E	F	G
1							
2		**VCAM-1**	**CM**	**10 ng/ml**	**50ng/ml**	**100ng/ml**	
3		Mean	1	1.776267	1.804204	2.047903	
4		Stdev	0	0.38739	0.849501	0.910706	
5				*	*	**	
6			**P value**	**0.0041**			
7							
8		**KDR**	**CM**	**10 ng/ml**	**50 ng/ml**	**100 ng/ml**	
9		Mean	1	1.427213	1.523068	1.67416	
10		Stdev	0	0.293508	0.385352	0.646593	
11					*	**	
12			**P value**	**0.003**			
13							
14		**PCDH12**	**CM**	**10 ng/ml**	**50 ng/ml**	**100 ng/ml**	
15		Mean	1	1.609732	2.667512	2.229016	
16		Stdev	0	0.489385	1.156681	0.936288	
17					***	**	
18			**P value**	**< 0.0001**			
19							
20		**FLT-1**	**CM**	**10 ng/ml**	**50 ng/ml**	**100 ng/ml**	
21		Mean	1	1.33514	2.120251	1.446049	
22		Stdev	0	0.316149	0.293138	0.325645	
23				*	***	**	
24			**P value**	**< 0.0001**			
25							
26		**PECAM-1**	**CM**	**10 ng/ml**	**50 ng/ml**	**100 ng/ml**	
27			1	1.147605	1.491689	1.33207	
28			0	0.05573	0.514759	0.154124	
29					*		
30			**P value**	**0.03**			

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